

Molecular Characterization of *BRCA1* as Candidate Gene Marker for Subclinical Mastitis in Dairy Water Buffaloes (*Bubalus bubalis*)

Cyndi C. Biendima^{1,§}, Sonny C. Ramos^{2,4},
Mary Rose D. Uy^{2,4} and Claro N. Mingala^{2,3,§*}

¹ Department of Biological Sciences, University of the Philippines Manila, Ermita, Manila

² Biosafety and Environment Section, Philippine Carabao Center, National Headquarters and
Gene Pool, Science City of Muñoz, Nueva Ecija

³ Department of Animal Science, College of Agriculture, Central Luzon State University,
Science City of Muñoz, Nueva Ecija

⁴ Department of Biological Sciences, College of Arts and Sciences,
Central Luzon State University, Science City of Muñoz, Nueva Ecija

§both authors contributed equally to the work

Subclinical mastitis (SCM), a major problem in dairy animals all over the world, is caused by intramammary infections with coagulase-negative staphylococci. It causes enormous losses for breeders since it suppresses the milk yield and quality; hence, it influences the national income of the country. It is related to low milk production, altered milk consistency, low protein content and high risk of contamination due to pathogenic organisms. The goal of the present study is to discover new information about the gene of interest, *breast cancer 1 (BRCA1)*, early onset in the water buffalo genome by examining the presence of single nucleotide polymorphisms (SNPs) in water buffaloes. DNA from milk samples was isolated from thirty female riverine-type water buffaloes. Somatic cell score (SCS) was calculated from somatic cell count for each sample. The results showed two animals reached greater than 5, 5.6 and 5.8 SCS, respectively, which is indicative of SCM infection. Multiple sequence alignment revealed that all samples were monomorphic for *BRCA1* gene. The genetic homology is undeniably high; however, it is not conclusive that the polymorphism found in cattle can also be found in water buffaloes. It is recommended that other single nucleotide polymorphisms in other exon should be studied. Also, the search for other candidate genes and larger sample size are essential to fully understand if these polymorphisms really affect the susceptibility of animals to SCM.

Key words: *BRCA1* gene, somatic cell, subclinical mastitis, water buffalo

INTRODUCTION

Subclinical mastitis (SCM) is common in dairy animals caused by intramammary infections (IMI) with coagulase-negative staphylococci (CNS) that leads to herd problems (Thorberg et al. 2009). It causes enormous losses for breeders and is of great economic importance to dairy

farmers since it reduces milk yield, causes undesirable changes in the milk's composition, and increases the costs associated with control strategies (Ramachandrainh et al. 1990; Abdel-Radyand Sayed 2009; Plozza et al. 2011). Many factors such as pathogens, genetic factors, poor management practice and health of the dairy animals cause mastitis and SCM (Yuan et al. 2012). The low quality of milk is due to the decrease of lactose, α -lactalbumin, and

*Corresponding author: cnmingala@hotmail.com

fat in milk due to the decreased synthetic activity in the mammary tissue (Yuan et al. 2012).

Somatic cells present in milk samples are mainly the milk-secreting lining epithelial cells that have been regularly shed from the lining of the gland and leukocytes that have entered the mammary gland in response to injury of infection. During the inflammation, majority of the somatic cells present are neutrophils due to its influx in response to the infection (Sharma et al. 2011).

With the advancement in technology and new discoveries in the field of molecular biology, artificial selection of animals or plants that are important agriculturally and economically are being done (Dohoo & Meek 1982). There are different polymorphisms present in genes, and sometimes particular polymorphisms of genes are used to associate them to important traits. Once these polymorphisms are identified and significantly associated with a trait, they can be used as genetic markers.

The *breast cancer 1 (BRCA1)* gene, a part of RING-type zinc fingers (RNF) gene family, belongs to a class of gene known as tumor suppressor genes, which encode for the protein that helps prevent cells from growing and dividing too fast. The protein is directly involved in the processes of DNA damage repair, cell cycle regulation, transcriptional regulation, and other pathways implicated in the maintenance of genome stability as it interacts with other proteins produced from *RAD51* and *BARD1* genes. Together with other tumor suppressors, DNA damage sensors and signal transducer, they form a large multi-subunit protein complex known as *BRCA1*-associated genome surveillance complex (BASC) (Wang et al. 2000).

Studies indicate that mutations in the gene encoding *BRCA1* were associated with high risk of breast cancer in humans, but studies in bovine subjects are not as extensive. The *BRCA1* sequence is also a better predictor of disease alleles (compared to polymorphism) than either the murine or the canine sequences (Yuan et al. 2012).

The goal of this study is to discover new information about the gene of interest, *BRCA1 early onset*, in the water buffalo genome, to learn more about the differences in the genetic make-up of the said gene between cattle and water buffalo. Other goals include determining if the *BRCA1* gene marker found, that is, single nucleotide polymorphism (SNP) in cattle, can be used as a gene marker in water buffaloes which is associated with SCM; calculating the Somatic Cell Score (SCS), and measuring the homology of *BRCA1* gene markers of cattle and water buffaloes if the identified marker is associated with SCM in water buffaloes.

The result of this study can be used preliminarily in detecting the presence of polymorphisms in water buffalo

that could further examine the association with SCM. Also, water buffalo is one of the important agricultural animals in the Philippines. However, compared to other dairy animals such as cattle, studies about water buffalo genome remain scarce. Hence, this study could supplement the current information in the water buffalo genome, as no study has been conducted on *BRCA1* gene in water buffaloes yet.

MATERIALS AND METHODS

Sample Collection

Milk samples were collected from thirty female riverine-type water buffaloes selected randomly from the herd of Philippine Carabao Center (PCC). Fifty milliliter-pooled milk were collected in the afternoon from each test subject and was stored in a 50-mL conical tube. The milk samples were labeled accordingly and were immediately kept in an insulated ice chest containing coolants to prevent somatic cell degradation. Milk samples were processed immediately in the laboratory for DNA extraction and PCR.

DNA Extraction from Milk Samples

DNA was extracted from the milk samples adapted from the protocol of Murphy et al. (2002). Milk samples were thawed at room temperature. Each conical tube containing 50 mL pooled milk was centrifuged at 2200 x g for 10 min. The upper fat layer was scraped while the supernatant was discarded, with 1 mL milk and pellet at the bottom of the tube. The milk was then transferred to a 1.5 mL microcentrifuge tube.

Casein in the milk sample was dissolved by adding 300 μ L EDTA (pH 8.0) and 200 μ L Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA (APS Chemicals Limited, NSW, Aus) at pH 7.6 to the microcentrifuge tube and was incubated for 10 min. The pellet in this solution was re-suspended by vortex mixing then centrifuged at 16000 x g for 20 sec. The supernatant was pipetted and discarded, with 100 μ L which contained the somatic cells. These cells were washed twice by adding 1.4 μ L TE buffer (Promega, Madison, Wisconsin, USA) and were incubated at room temperature for 10 min, then re-suspended and centrifuged afterwards. The supernatant was discarded following the protocol mentioned above. Then, 100 μ L supernatant was added to the 100 μ L digestion solution with 40 mM Tris-HCl (Promega, Madison, Wisconsin, USA) at pH 8.3, 50 mM KCl (Techno Pharmchem, Delhi, India), 3 mM MgCl₂, 1 μ L of Tween 20 (Sigma-Aldrich, USA), and 20 μ L of Proteinase K (Promega, Madison, Wisconsin, USA) at 20 mg/mL and incubated at 55°C for 3 hrs in a heat block.

DNA purification was done by adding 200 μ L chloroform (Sigma-Aldrich, USA) to the tube, and then gently mixed by inverting several times. It was then centrifuged at 4700 x g for five min. Approximately 200 μ L of the upper aqueous solution containing DNA sample was pipetted and transferred to a new tube, leaving the chloroform and other debris behind. Four hundred microliter of 95% ice-cold ethanol (ThermoFisher Scientific, Singapore) was added to the new tube, then it was inverted several times. Samples were incubated for 10 min at -20°C to allow DNA precipitation. It was centrifuged at 8000 x g for 5 min, and then the ethanol was decanted. Then, 200 μ L TE buffer, 300 μ L 5M sodium acetate (Techno Pharmchem, Delhi, India), and 1 mL 95% ethanol was added to resuspend the DNA pellets. Samples were again incubated, centrifuged, decanted and resuspended following the protocol.

After the final decantation, the DNA sample adhering to the walls of tube was dried at room temperature until it is completely dehydrated. Fifty microliters of DNA rehydration solution was added to rehydrate the DNA sample.

Amplification and Sequencing of *BRCA1* Gene

The primers used were adapted from the study conducted by Yuan et al. (2012) which was used in cattle. The forward (5'-TGCAGTGGAAATTCCAAATAAACT-3') and reverse (5'-GAATTAGATCTTCAGCTATGTGGC-3') primers had the expected amplicon size of 209 bp which is located in exon 9. After DNA extraction, a total of 20 μ L reaction mixture was prepared containing more than 50 ng genomic DNA, 1x PCR Buffer (Promega, Madison, Wisconsin, USA), 0.25 μ M each of forward and reverse primers, 6 μ M MgCl₂ (Promega, Madison, Wisconsin, USA), 0.25 mM dNTPs (iNtRON Biotechnology, Lynnwood, WA) and 0.05 U *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA). The thermal cycling process (ESCO Healthcare, Singapore Ltd.) was composed of the initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 seconds and extension at 72°C for 45 seconds. The terminal extension step was done at 72°C for 8 min.

After PCR amplification, 3 μ L of PCR products were electrophoresed in 2% agarose gel containing GelRed™ Nucleic Acid Stain (Biotium, Fremont, CA) and 1x TAE (Sigma-Aldrich, USA) as running buffer at 110 volts for 30 min and visualized using gel documentation system (ProteinSimple, CA, USA). Presence of 209 bp bands indicated successful amplification of the *BRCA1* gene. Amplified PCR products were submitted to 1stBASE Laboratories (Sdn Bhd., Malaysia) to evaluate the DNA sequences of the water buffalo samples with the *BRCA1* gene of cattle as a reference. These sequences were then analyzed by means of multiple sequence alignment using MEGA version 5 (Tamura et al. 2011).

Computation of SCS via Somatic Cell Count (SCC)

Data regarding the SCC of each water buffalo included in this study was obtained from the Genetic Improvement Program Unit of PCC, by testing the milk in monthly basis for its fat, protein content and somatic cell counting. The SCS (Ødegård et al. 2003) of each sample was calculated using the formula, wherein SCC is somatic cells per milliliter:

$$SCS = \log_2\left(\frac{SCC}{100000} + 3\right).$$

RESULTS

Somatic Cell Score

The average SCC of the water buffaloes is 156,032 cells/mL ranging from 20,940 cells/mL to 694,410 cells/mL, while the average calculated SCS is 3.04±1.33 ranging from 0.74 to 5.8. Only two animals reached greater than 5 SCS, 5.6 and 5.8, indicative of SCM infection.

PCR Amplification of *BRCA1* gene

The isolated genomic DNA samples from thirty lactating riverine-type water buffaloes showed that *BRCA1* primers adapted from cattle worked on them by the presence of 209 bp band (Figure 1).

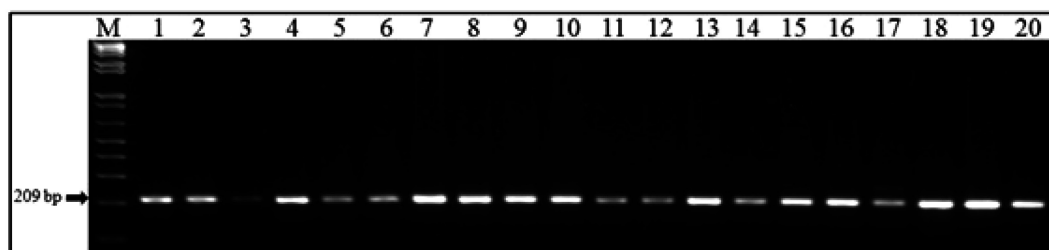


Figure 1. BRCA1 primer designed for cattle recognized complementary sequence in water buffalo genome with an amplicon size of 209 bp band. M = 1 kb plus ladder; Lanes 1-20 = samples

Due to the presence of bands, it is expected that the homology between the cattle and water buffalo DNA sequences are high (El Nahas et al. 2001). Result of analysis showed that the DNA homology between the two animals is 99%.

All of the 30 samples have the length of 209 bp which is the same with cattle, and only three bases differ between cattle and water buffalo. These differences are G, C and T in cattle that are C, A and C in water buffalo at positions 23, 115, and 136, respectively with the complete amplicon sequence of cattle and water buffalo (Figure 2). Likewise, those 30 samples possess the same DNA sequence. The remarkable difference in position 23 which is G for cattle and C for water buffalo is due to the intentional substitution in primer used by Yuan et al. (2012).

However, the SNP (A→T) that is present in cattle at position 25 was not found in the water buffaloes. All of the 30 samples have the same base regardless of their SCS values.

DISCUSSION

The cattle genome is well studied in both physical gene mapping and molecular characterization, and many studies reported the great similarity between the banding patterns of cattle and water buffalo chromosomes. With this, it is expected that there is chromosome conservation between the two species and high genetic homology (El Nahas et al. 2001). In this study, it is not surprising that the *BRCA1* primers which are originally designed

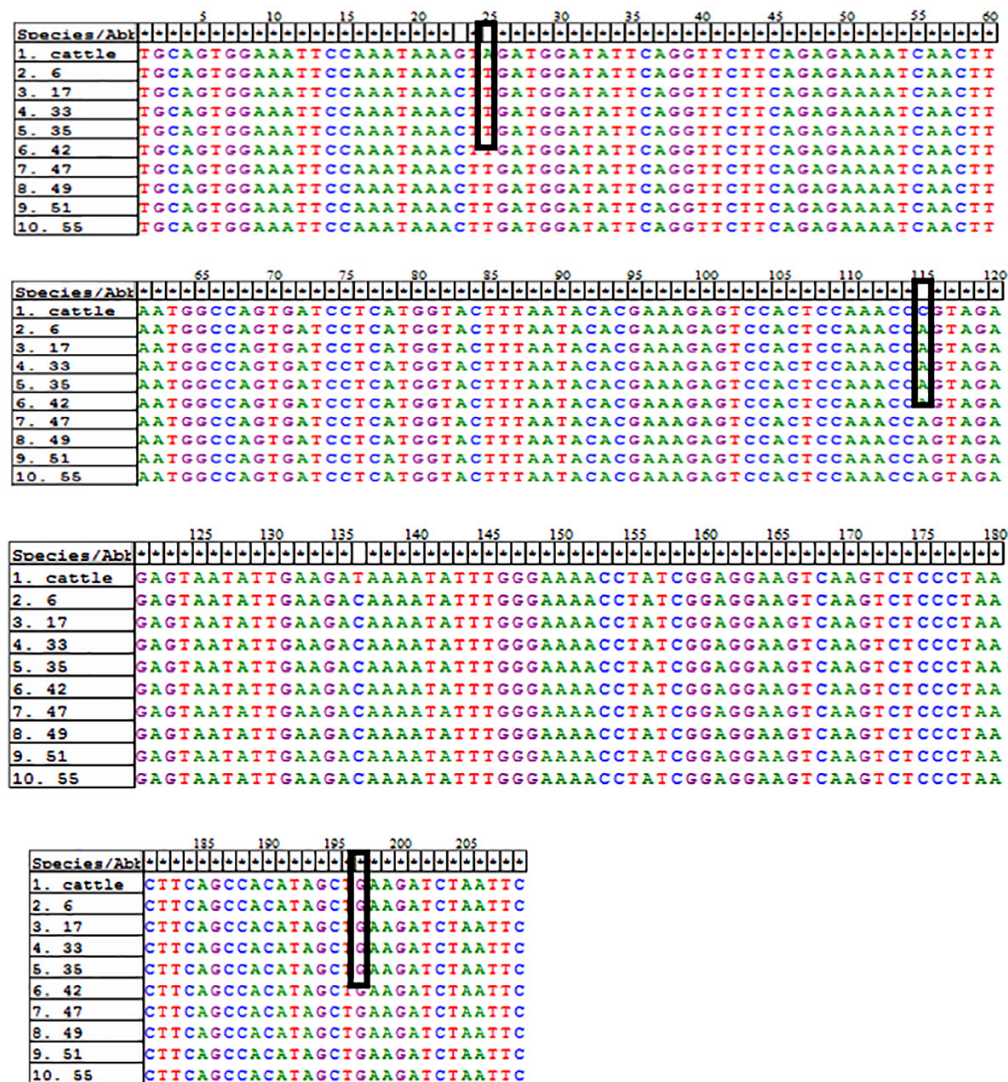


Figure 2. Comparison of cattle *BRCA1* sequence from GenBank and sequences of *BRCA1* amplified products of water buffalo. Three nucleotide differences were identified in positions 23, 115 and 136 which indicates that *Bubalus bubalis* belongs to a different genus.

for cattle will also work on water buffalo. Also, the use of tandemly repeated short DNA sequences and highly polymorphic microsatellite markers for cattle are widely used in the characterization of water buffalo. These repeat-flanking sequences of microsatellite loci are also often conserved among the closely related species, thus cross species amplification on related species for which the microsatellite markers have not been yet developed are useful. In a study of Nagarajan et al. (2009), 594 cattle microsatellite primer pairs were tested if it will work on water buffalo genomic DNA. Four hundred fifty seven (76.9%) were amplified and gave discrete amplification products.

The homology between cattle and water buffalo *BRCA1* gene is undeniably high (99%) with 206 of 209 matched pairs according to NCBI's BLAST, however, it is not enough to ensure that the polymorphism found in cattle will be exhibited by the water buffalo as well. Therefore, despite of very close homology of the two species, it is still not conclusive that polymorphism found in cattle can also be found in water buffaloes.

Regardless of the health condition of the animals included in this study based on their SCS, the DNA sequences of their amplicons are just the same which corroborates with chromosome conservation between cattle and water buffalo. There are two samples included in this study that has >5 SCS value, meaning those animals were more likely to be infected with SCM during sample collection. Despite of that, those two samples also showed the same genotype like the rest of the samples. These further validate the homology between the two species regardless of SCS value. Also, the three animals included in this study have an SCS value <1, meaning they are more likely to have a healthier condition than those which had higher values, but still they have the same genotype.

Several studies regarding the polymorphisms of cattle genes were adapted in water buffaloes, however, in some, monomorphism was observed. As mentioned earlier, of the 497 amplified microsatellites in the study of Nagarajan et al. (2009), 391 (85.5%) of these were polymorphic while the remaining 66 (14.5%) were monomorphic. There was also a study for the cattle *diacylglycerol acyltransferase (DGATI)* gene using PCR-RFLP which showed two patterns as the result of non-conservative polymorphism from the ancestral Lysine variant, associated with high milk fat content, to alanine variant which is associated with high milk yield.

However, when this study was adapted in water buffalo, only the lysine variant was present among the 41 samples included in the study (Ozdil & Ihlan 2012). The investigation about water buffalo *Oxidized Low Density Lipoprotein Receptor 1 (OLRI)* gene, the gene responsible

for coding the major protein that binds, internalizes, and degrades oxidized low-density lipoprotein, was also carried out using PCR-RFLP and was adapted from the established gene marker from cattle, but still monomorphic patterns were observed. Further study between the *OLRI* amplicon sequence of cattle and water buffalo showed nine nucleotide variations out of the 288 bases (Deshpande et al. 2013). Another investigation that yielded the same result with this study is the association of κ -casein (*CSN3*) gene originally from cattle that were adapted in water buffalo. This gene encodes for a sub-unit of casein, a major milk protein which comprises approximately 80% of the total protein content of the milk. In cattle, A and B variants are present and they differ in two amino acid at positions 136 and 148, in which variant B is more preferred due to its association with the greater yield in cheese making and has a direct effect on manufacturing process of dairy products. When these polymorphisms were studied in 115 water buffaloes using PCR-SSCP, monomorphic pattern was also observed and it was found out that the only present allele in the selected sample size is the B variant (Ontaviano et al. 2005).

With these findings, *BRCA1* gene exhibited monomorphism across the samples with or without subclinical mastitis since it is present only in homozygosis. The polymorphism information content (PIC), effective number of alleles (N_e) and Hardy-Weinberg equilibrium cannot be calculated as well.

It is also important to note that one of these mismatched pair is due to the intentional substitution of Yuan et al. (2012) in the forward primer they used from the nucleotide G to C so that the restriction site will appear as they used PCR-RFLP which utilizes the capability of several restriction enzymes to recognize specific palindromic sequences and to cut them into several fragments, depending on the number of the restriction sites available. However, even with this modification, the restriction site is still not available in water buffalo because *MaeI*, the restriction enzyme used, recognizes the sequence CT[^]AG, on the other hand, in all of the water buffalo DNA samples used in this study, the sequence for the supposed to be location of that restriction site is CTTG.

CONCLUSION AND RECOMMENDATIONS

All of the 30 water buffaloes used in the study showed monomorphism when their amplified products were sequenced, having 99% genetic homology compared to cattle. Even though it is undeniably high, it is still not an assurance that polymorphism found in cattle can also be found in water buffaloes. It would be better if other

SNPs in other exons will also be studied in *BRCA1* as well as other candidate genes. Likewise, a bigger sample size will be more helpful to fully understand if these polymorphisms really affect the susceptibility of animals on SCM.

ACKNOWLEDGMENT

The research team extends their utmost gratitude to Dr. Arnel N. Del Barrio, Philippine Carabao Center (PCC) Executive Director, for his unrelenting support in this study. Also, special thanks to all the research staff of the Biosafety and Environment Section of PCC for their technical support.

REFERENCES

- ABDEL-RADY A, SAYED M. 2009. Epidemiological studies on subclinical mastitis in dairy cows in Assiut Governorate. *Vet World* 2(10):373–380.
- DESHPANDE M, RANK D, VATALIYA P, JOSHI C. 2013. Oxidized low density lipoprotein receptor 1 (OLR1) gene polymorphism in Mehsana water buffaloes (*Bubalus bubalis*). *Buff Bull* 32(4):260–264.
- DOHOO IR, MEEK AH. 1982. Somatic cell counts in bovine milk. *Can Vet J* 23(4):119–125.
- EL NAHAS S, DE HONDT H, WOMACK J. 2001. Current status of the riverwater buffalo (*Bubalus bubalis* L.) gene map. *J Heredity* 92(3):221–225.
- MURPHY MA, SHARIFLOU MR, MORAN C. 2002. High quality genomic DNA extraction from large milk samples. *J Dairy Res* 69(4):645–649.
- NAGARAJAN M, KUMAR N, NISANTH G, HARUBASKAR R, PARANTHAMAN K, GUPTA J, MISHRA M, VAIDHEGI R, KUMAR S, RAJAN A, KUMAR S. 2009. Microsatellite markers of water buffalo, *Bubalus bubalis* – development, characterization and linkage disequilibrium studies. *BMC Genet* 10:68.
- ØDEGÅRD J, JENSEN J, MADSEN P, GIANOLA D, KLEMETSDAL G, HERINGSTAD B. 2003. Detection of mastitis in dairy cattle by use of mixture models for repeated somatic cell scores: A Bayesian approach via Gibbs sampling. *J Dairy Sci* 86: 3694–3703.
- ONTAVIANO A, TOHATI H, SENAJ, MUNOZ M. 2005. Kappa-casein gene study with molecular markers in female water buffaloes (*Bubalus bubalis*). *Genet Mol Biol* 28(2):237–241.
- OZDIL F, ILHAN F. 2012. DGAT1 – exon 8 polymorphism in Anatolian water buffalo. *Livest Sci* 149(1–2): 83–87.
- PLOZZA K, LIEVAAT JJ, POTTS G, BARKEMA HW. 2011. Subclinical mastitis and associated risk factors on dairy farms in New South Wales. *Aust Vet J* 89:1–2.
- RAMACHANDRAIN K, KUMAR KS, SRIMANNARANA O. 1990. Survey of mastitis in pure Jersey herd. *Indian Vet J* 69:103.
- SHARMA N, SINGH NK, BHADWAL MS. 2011. Relationship of somatic cell count and mastitis: An overview. *Asian Australas J Anim Sci* 24(3):429–438.
- TAMURA K, PETERSON D, PETERSON N, STECHER G, NEI M, KUMAR S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739.
- THORBERG BM, DANIELSSON-THAM ML, EMANUELSON U, PERSSON WALLER K. 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *J Dairy Sci* 92:4962–4970.
- WANG Y, CORTEZ D, YAZDI P, NEFF N, ELLEDGE SJ, QIN J. 2000. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Gene Dev* 14(8): 927–939.
- YUAN Z, CHU G, DAN Y, LI J, ZHANG L, GAO X, GAO H, LI J, XU S, LIU Z. 2012. BRCA1: A new candidate gene for bovine mastitis and its association analysis between single nucleotide polymorphism and milk somatic cell score. *Mol Biol Rep* 39(6): 6625–6631.